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**14. ABSTRACT:** Parkinson Disease (PD) is the second most common neurodegenerative disease of our aged population behind Alzheimer's Disease. Epidemiological, animal and cell culture studies have shown that inflammation is a part of the PD morphological picture. It has been suggested that α-synuclein (a major component of the Lewy bodies present within dopamine neurons of the PD substantia nigra {SN}) is responsible for the observed inflammatory response in the PD brain. We injected α-synuclein and mutated synuclein into the substantia nigra (SN) of rats (8 ug/4ul). We also performed cell culture studies on the activation of microglia by alpha-synuclein. In our *in vivo* studies, we found that both alpha-synuclein and A53T mutated synuclein both caused apomorphine-induced rotations. The number of rotations were small but significant and indicates that the synucleins caused an increase in sensitivity of the dopaminergic (DA) receptors in the SN. These studies indicate that there is some interaction between DA and the synucleins. In our *in vitro* studies, we noted that the synucleins caused microglia to adhere to plastic surfaces. This adhesion was blocked by EDTA which indicated that the process of adhesion in this case was calcium-dependent. In our examination of cell migration using various synucleins, we found that only LTB4, a leukotriene, and CX3CL1, a chemokine, enticed microglia to migrate to naked or laminin-coated filters. While we cannot conclude from our cell culture studies that synucleins can trigger neuroinflammation, we can state that the synucleins can activate microglia and cause them to migrate.

**15. SUBJECT TERMS**
Alpha-synuclein, glia, neuroinflammation, Parkinson Disease

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## Introduction

Parkinson's Disease (PD) is a common progressive neurodegenerative disorder characterized clinically by resting tremor, slowness of movement, muscle rigidity and postural instability (Fahn and Przedborski, 2009), all attributed to, mainly though not exclusively, the loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and their dopaminergic terminals in the corpus striatum of the nigrostriatal pathway in the brain (Fahn and Przedborski, 2009). The profound loss of DA in the striatum accounts for the noted motor deficits (Hornykiewicz and Kisk, 1987). Of the various current therapies including dopaminergic agonists and cholinergic antagonists, the most reliable and most common therapy still remains Levodopa (L-DOPA), a precursor for dopamine (DA) (Fahn and Przedborski, 2009). Pathologically, aside from the loss of the DA neurons in the SNpc, PD is characterized by the presence of interneuronal inclusions called Lewy bodies (Schultz, 2006), found within dopaminergic neurons of the SNpc. These bodies are eosinophilic cytoplasmic aggregates composed of a variety of proteins, including α-synuclein, ubiquitin, parkin, and neurofilaments and are necessary for the definitive diagnosis of PD (Shultz, 2006). One protein in the Lewy body, α-synuclein, is well-conserved across mammalian species (Maroteaux et al, 1988), is conspicuously expressed throughout the brain and has been identified in close proximity to synaptic vesicles in presynaptic terminals (Maroteaux et al, 1988; Iwai et al, 1995). To date, three missense mutations in α-synuclein (A53T, A30P and E46K) have been noted and these mutations as well as the overexpression of wild-type α-synuclein have been linked to the familial form of PD (Polymerpoulos et al, 1997; Kruger et al, 1998; Masliah et al, 2000; Zarranz et al, 2004;) and, more importantly, to an early-onset form of the disease. Both the A53T and the A30P mutants increase the rate of α-synuclein fibrillation (Bennett, 2005) and the formation of β-sheets (Narhi et al, 1999) while, the overexpression of WT α-synuclein in a number of cell types can result in aggregate formation (Lee and Lee, 2002). Post-translational modification of WT α-synuclein, such as nitration (Przedborski et al, 2001) and oxidation (Hashimoto et al, 1999), can lead to structural changes in the cells, and if these are not properly cleared, they may give rise to a neurotoxic event within the cells.

Neuroinflammation is now recognized as being very much a part of the PD morphological picture (McGeer and McGeer, 2008). Furthermore, glial activation is thought to be a contributor to the progression of DA neuron degeneration in PD (Fahn and Przedborski, 2009). The question is, however, how are glial cells activated in PD? Theory has it that the observed activation of glial cells in neuroinflammation may be the cause of the progressive nature of the disease, but this remains to be clarified. We do know that, on autopsy, activated glial cells are present in the SNpc of PD brains and there seems to be evidence of an on-going active degenerative process in this brain area (Langston et al, 1999). We also know that pro-inflammatory components, such as interleukin 1β (IL-1β) and prostaglandin $PGE_2$, are increased in the SNpc and in the CSF from PD patients (Mogi et al, 1994, 1996, 2000). In PD models, activated glia are present in the SNpc, along with markers of inflammation, such as elevated levels of NADPH oxidase (Wu et al, 2002), inducible nitric oxide synthase (iNOS) (Liberatore et al, 1999) and $PGE_2$ (Teismann et al, 2003). Our interesting finding of the presence of post-translational modified α-synuclein (Przedborski et al, 2001) within the DA neurons of the SNpc points to the possibility that a connection exists between this finding, oxidative stress and neuroinflammation as nitrated synuclein is most likely a permanent fingerprint whose insolubility may initiate the noted neuroinflammation. Previous work with an injection of neuromelanin into the SNpc of rats that

resulted in an inflammatory response plus increased synuclein and ubiquitin accumulation within the DA neurons of the SNpc (Jackson-Lewis et al, 2008) demonstrates that if enough of an accumulation of synuclein occurs within the DA neuron, this may cause the cell to rupture, spewing its contents including the synucleins in their various forms and modifications, into the extracellular space. And, it has been demonstrated, at least in the test tube, that synucleins can activate microglial cells via some signaling pathway.

**Body of Research**

Our overall goal is to examine and establish a relationship between neuroinflammation and the synucleins since there is strong evidence that α-synuclein is involved at some level in all cases of PD as it is a major component of the Lewy body. Recent evidence, both in vitro and in vivo, suggest that the varied forms of synuclein can initiate inflammation and can also kill DA neurons. What is so intriguing about this protein is that synuclein is a normal component of the cell, yet it can be toxic to the cell. In studies with synuclein, the inflammatory response is thought to be related to microglial activation. Evidence for this comes from three different areas of research: epidemiological, cell culture, and animal models. Epidemiology studies show that taking ibuprofen on a regular basis decreased the risk of developing PD by 35% (Chen et al, 2005). Cell culture studies have demonstrated that exogenously applied, aggregated α-synuclein activates microglia which become toxic to DA neurons (Zhang et al, 2005). Animal models have produced several pieces of interesting data. For example, the MPTP mouse model indicates that inflammation in the SNpc appears to be self-sustaining while the α-synuclein model indicates that overexpression of this endogenous protein can provide a source of inflammatory response (McGeer and McGeer, 2008). Moreover, both cell culture studies and animal studies have shown indirectly that DA neurons are highly sensitive to inflammation and that microglial cells in the vicinity of DA neurons are activated prior to their attack on DA neurons (Fernogut and Chesselet, 2004). Given the fact that a significant amount of evidence points to involvement of microglia in the inflammatory response seen in PD and α-synuclein can activate microglia, our efforts are geared toward trying to establish the mechanisms by which α-synuclein initiates an inflammatory response. Thus, to investigate the possible link between neuroinflammation and the synucleins in terms of PD, we have proposed the following specific aims.

**Specific aim-I. Assess the effects of extracellular α-synuclein on glial mobility and proliferation.**

***Hypothesis:*** Post-translationally modified and mutated α-synuclein species provoke a greater clustering of glial cells around the site of intracerebral injection than wild-type soluble α-synuclein. In magnitude, this inflammatory response parallels the known pathogenicity of a range of different α-synuclein species, and is due to a combination of key features of the glial response, namely proliferation and chemotaxis.

***Plan*:** (**A**) Rats will receive into the substantia nigra a single injection of modified (fibrillar, nitrated, or oxidized) or undified wild-type or PD-linked mutant α-synucleins. Brains will then be harvested and glial cell morphology, topography and density will be compared among each different group. (**B**) Glial cultures will be exposed to the same set of α-synuclein species and their effects on glia chemotaxis and proliferation will be compared.

**Specific aim-II. Examine the stability of extracellular α-synuclein and its effect on glial cell activation.**

***Hypothesis:*** Modified and mutated α-synuclein species are not cleared (or engulfed) by glial cells as efficiently as is WT soluble α-synuclein and are more potent than wild-type soluble α-synuclein in stimulating the production of a variety of factors such as reactive oxygen species (ROS) or nitric oxide (NO) from glial cells.
***Plan*:** (**A**) The stability and phagocytosis of extracellular α-synuclein species will be compared *in vivo* (using the SA-IA model) and *in vitro* (using primary glial cultures) by immunostaining for α-synuclein and glial markers and confocal microscopy, and by an *in vitro* protein fragmentation assay. (**B**) The effect of the α-synuclein species on the *in vivo* and *in vitro* production of chemokines, cytokines, ROS and NO by glia will also be compared.

**Specific aim-III. Define the mechanism by which glial cells recognize α-synuclein.**
***Hypothesis*:** Extracellular unmodified, modified and mutated α-synuclein species interact with glia through specific cell surface receptors, leading to glial cell activation. Moreover, modified and mutated α-synuclein species are more potent in activating immune cells in the brain than wild-type soluble α-synuclein.
***Plan*:** (**A**) Identify the glial receptors involved in the recognition of the α-synuclein species. (**B**) Characterize the effect of abrogating (knockout mice) and inhibiting (blocking antibodies) glial receptors on endocytosis of and activation by α-synuclein species.

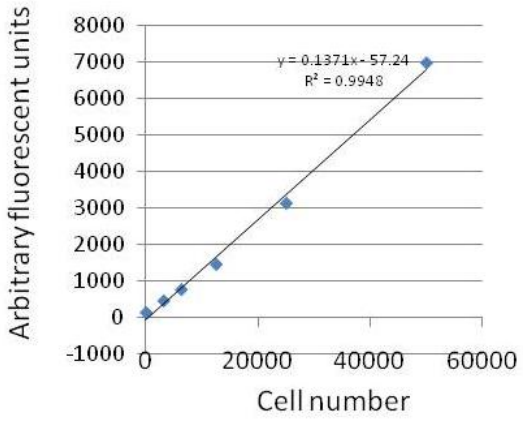
## Key Accomplishments

### Specific Aim I.

**In vivo Studies**: We have started to cut the perfused brains (time course) from WT- and A53T-injected rats for immunostaining for TH, Nissl and glia that were stored at -80°C. There are 48 brains in each set, n=6-8 brains per timepoint. We are collecting SNpc and striatum for immunostaining.

**In vitro studies:** we have made considerable progress in examining how microglial cells interact with extracellular matrices containing native synuclein, nitrated synuclein and mutated (A53T, A30P) synuclein. Specifically, we have developed an adhesion assay that rapidly and accurately assesses the number of cells adhering to a surface within a two hour incubation period using the InVitrogen CyQuant monitoring system. Basically, the CyQUANT® Direct Cell Proliferation Assay kit consists of two components: a green fluorescent nucleic acid stain and a background suppression dye. We have been using BV2 murine microglial cell lines for our adhesion assay. We allow the cells to adhere to various surfaces and then we wash away the non-adherent cells and lyse the cells using a lysing buffer and incubation at -80$^{o}$ C for 20 min. The CyQUANT dye is then added and upon binding to the nucleic acids - a green fluorescent signal that corresponds to the number of adherent cells. This signal is read on our Cytoflour II cell plate reader.

Fig. 1 – Standard curve




2. Which is the best extracellular matrix to measure BV2 adhesion?

We examined a variety of matrices by coating non-tissue culture plates (96 well plates) with either poly-D-Lysine (1ug/well) and then adding various proteins such as Collagen IV, Laminin, or bovine serum albumin. In addition, we coated the cells with Collagen IV, Laminin, or bovine serum albumin in the absence of Poly-D-Lysine. In summary, we found that the most consistent results and the greatest plating efficiency occurred with wells coated initially with Poly-D-Lysine and then with murine Laminin. More than 90% of the cells were adherent after a 2 hour incubation at 37$^{o}$C. We also examined the effects of different types of culture media and discovered that the best medium for maintaining and growing these cells was INDM supplemented with 10% fetal bovine serum.

3. Effects of native synuclein, nitrated synuclein and mutated synuclein on the capacity of cells to adhere to laminin-coated surfaces.

BV2 cell are normally maintained as adherent cultures on 75 mm culture flasks. To remove these cells, the cells are first washed with PBS and then incubated at 37$^{o}$C for 5 mins in PBS containing 0.025% trypsin and 10 mM EDTA. Serum-containing medium is then added to the detached cells to inactivate the typsin and the cells are washed twice in complete INDM media. 50,000 BV2 cells were added to each Laminin-coated well and allowed to adhere for 2 hr at 37$^{o}$C in the presence or absence of 5 mM EDTA. At the end of the incubation period, the cells were washed three times with phosphate buffered saline and the number of adherent cells was assessed using the CyQUANT assay. We varied the concentration of Poly-D-Lysine and laminin to optimize our assay. The best conditions were to coat the wells first with 1ug/ well of Poly-D-Lysine followed by a second coating with murine laminin at 5ug/well. Under these conditions, we observed that the wells coated with a lower coating of laminin and an additional coating of native synuclein (5 ug/well) significantly enhanced adherence of BV2 microglia (Fig. 2). In addition, we observed that both nitrated synuclein and mutated synuclein further enhanced the adherence of BV2 microglial cells to these surfaces.

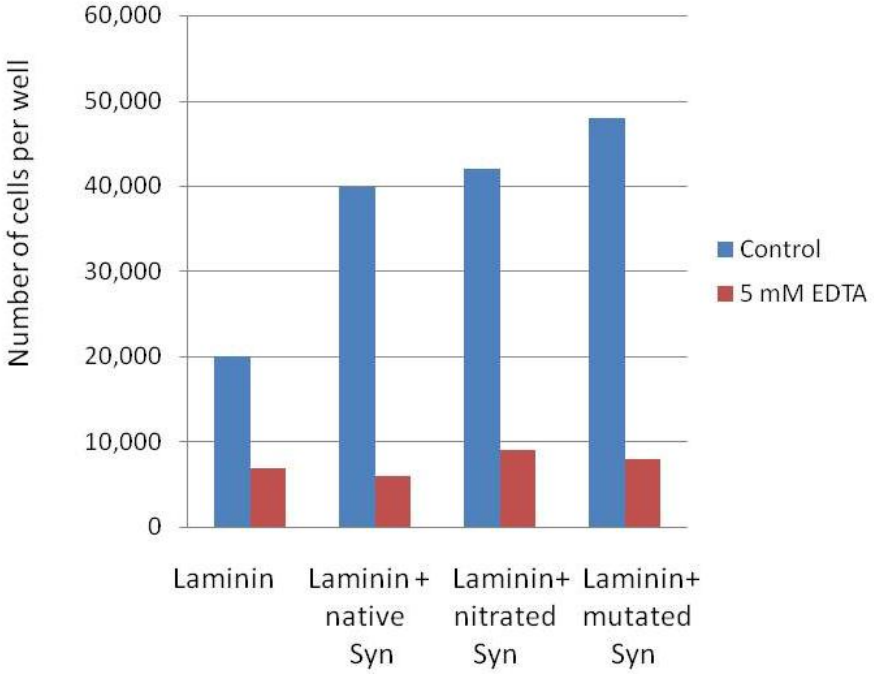
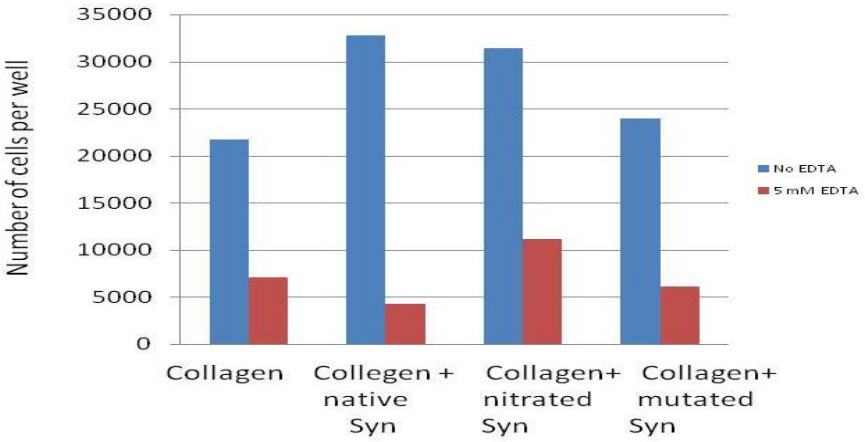



Fig. 2. Cell adhesion to plastic surfaces coated with Laminin alone, with Laminin and native synuclein (Syn), nitrated Syn or mutated Syn. 50,000 cells were allowed to adhere for 120 min in INDM medium supplemented with 10% fetal bovine serum in the presence or absence of 5 mM EDTA. Cell adhesion per well was assessed by CyQUANT assay.

As a first step in trying to identify the specific cell surface proteins mediating adhesion, we examined the effects of EDTA which chelates $Ca^{++}$ and $Mg^{++}$. In general, cell surface receptors that mediate cell adhesion can be divided into two classes. A) integrins that require divalent cations and B) other plasma membrane proteins such as pattern recognition proteins and Toll receptors that mediate adhesion in a $Ca^{++}$ independent manner. Our results (Fig 2) demonstrate that the presence of EDTA dramatically inhibits BV2 cell adhesion to all surfaces. To further demonstrate that integrins mediate BV2 adhesion, we also examined cell adhesion to Collage IV which is another established matrix whose adhesion is mediated by beta 1 integrins. As expected, the presence of EDTA dramatically inhibited cell adhesion to Collagen surfaces as well (Fig. 3).




Cell Migration: We have attempted to develop a good model to assess BV2 microglial cell migration across naked 8 um cell culture inserts or inserts coated with either laminin, collagen, or fibrinogen. These cells did not appear to chemotax across naked 8 um inserts within 24 hr or across protein coated inserts in response to LTB4, to MCP-1, or to CX3CL1. It appears that only to LTB4 and CX3CL1 did the cells migrate to the other side of the naked or laminin-coated filters. We need to confirm this finding using fluorescence-labeled BV2 cells. These cells can be labeled with 1.5 uM of BCECF dye.

We have also acquired beta- and gamma-synuclein to compare to the results obtained with α-synuclein in cell culture.

**Specific Aim II**

**In vivo studies**: we are now optimizing immunostaining for human α-synuclein in order to distinguish the injected synucleins from rat synuclein. We have also lesioned rats with WT and A53T mutated synucleins and rotated these synuclein-lesioned rats to amphetamine (Figure 4A; ipsilateral rotation) and apomorphine (Figure 4B; contralateral rotation) as a first step in gauging the extent of lesion damage.

Figure 4A .

Wild-Type A53T

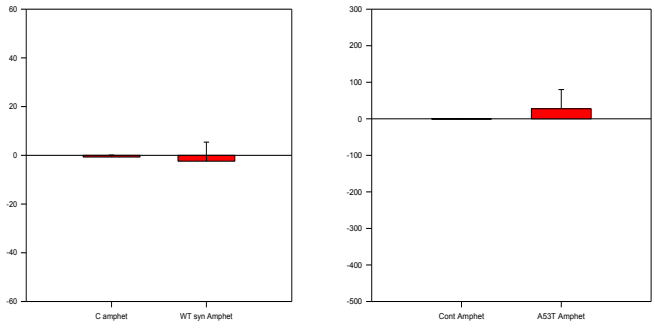
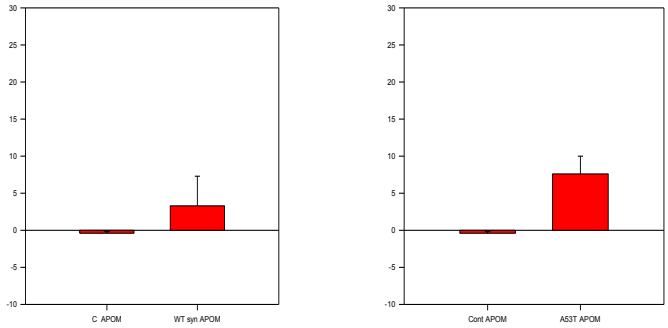


Figure 4B. Wild-type A53T




The same aforementioned rats have been euthanized and left and right striata and left and right SN (stored at -80°C) will be used to measure dopamine and metabolites by HPLC. We are now in the process of preparing left and right rat striata and SN from rats lesioned with WT α-synuclein and A53T mutated synuclein euthanized at selected time points for western blot analyses for inflammatory markers. Samples are the same timepoints as in Specific Aim I. **Please note that Dr. Jackson-Lewis did not work on this project for 10 -12 weeks as she had spinal surgery in April 22, 2010 and returned to work 7 July, 2010.**

**Specific Aim III**

We have stabilized our synuclein knockout mouse colony and are now in the process of acquiring and breeding integrin knockout mice for these studies.

**Reportable Outcomes**

We are now in the process of laying out what would be the first publication for this study.

**Conclusions**

Aside from increased oxidative stress, mitochondrial dysfunction, abnormal protein aggregation, and failure of the ubiquitin-proteosome system, neuroinflammation is very much a part of the PD picture (Przedborski, 2010). How it is initiated and what is its role in PD is unknown, although there is some speculation that the inflammation seen in the SN of PD patients is responsible for the progressive nature of the disease (Przedborski, 2010). Recent evidence suggests that α-synuclein may actually be an initiator of noted inflammation (Sulzer, 2010). In fact, in our pilot study, we injected a high concentration (5ug/microliter, total volune 4 microliters) wild-type (WT) α-synuclein and, two days later, observed iba-1 positive cells, indicative of activated microglia, in the SN of the α-synuclein-injected rats. Because the 5ug/ul dose actually put a hole in the SN, we injected a new batch of rats with 2ug/ul for a total of 8 micrograms in a total volume of 4 microlitres. We also injected mutated A53T α-synuclein at the same concentration and in the same volume. While we have not immunostained these samples for an inflammatory response yet, we did perform rotational behavior studies on both WT and A53T SN-injected rats prior to sacrifice. We did see that amphetamine did not elicit ipsilateral rotational (to the same side of the lesion) behavior in either WT or A53T unilateral SN-lesioned rats, whereas

apomorphine, in both situations, did cause contralateral (away from the side of the lesion) rotations. Amphetamine has been used to give some indication of a left/right dopamine (DA) imbalance in the nigrostriatal system (Dougherty, 1981), but a more exact examination of left/right brain DA difference in the striatum can be seen on HPLC analyses. The fact that apomorphine produced a small but significant number of rotations away from the lesioned side demonstrates sensitivity of the DA receptor (Traub et al, 1985), and thus, a small DA deficit. We will know this for sure when we analyze the samples for monoamine levels.

The fact that we do see rotational behavior suggests some level of toxicity from the synuclein lesioning. The original SN injections (5 ug/ul) elicited microglial activation as demonstrated on iba-1 immunostaining. We expect a glial response, but of less magnitude with the decreased dose. Since the inflammatory response was a result of the synuclein injection, this suggests that synuclein may be, at least in part, responsible for this response. Recent cell culture experiments seem to point us in the right direction for the answer as it has been shown that human BE-M17 neuroblastoma cells overexpressing WT, A53T or A30P α-synuclein, in the presence of iron or free radical generators such as dopamine or $H_2O_2$, exhibited iron-induced aggregates that were synuclein and ubiquitin positive. Toxicity of these afore-mentioned synucleins was increased 4-fold (Osterova-Golts et al, 2000) with a rank order for both cytotoxicity and aggregate formation of A53T being more toxic than A30P which was more toxic than WT α-synuclein. Furthermore, Zhou et al (2002) showed that, in primary cell cultures derived from the embryonic human mesencephalon overexpressing either WT or A53T α-synuclein, there was a significant reduction in the number of DA cells with the mutant α-synuclein exhibiting more toxicity than the WT.

In vivo models have also connected DA cell loss to α-synuclein. In a mouse model overexpressing WT α-synuclein, Masliah et al (2000) found cytoplasmic α-synuclein-positive inclusions, motor deficits and DA neuron loss. Gomez-Isla et al (2003), in their A30P transgenic mice, found a progressive motor disorder, α-synuclein accumulation and a massive gliosis in the central nervous system but saw no loss of DA neurons. Furthermore, in viral vector studies, recombinant WT and A53T synucleins, delivered to the SN of adult mice, induced α-synuclein-containing inclusions, motor dysfunction and DA neuron degeneration (Gomez-Isla et al (2003). One of the most interesting studies tying α-synuclein to DA neuron degeneration and fitting with the PD picture was that of Klein et al, (2002) who noted that one year after exposure of the adult mouse SNpc to a viral vector containing A30P, 50% of the DA neurons were lost, but there was no motor deficit. These studies fit with the human condition of PD as no motor deficits are seen prior to a greater than 60% loss of DA neurons in the SNpc (DiMonte et al, 2000) and point to a toxic role played by α-synuclein in DA neurodegeneration.

Ever since Langston et al (1999) found an active glial process in the brains of individuals who had injested MPTP, increasingly researchers have sought to establish a role for inflammation in PD. At present, it is thought that when DA neurons degenerate, these Lewy body-containing cells release their Lewy body contents, primarily synucleins (WT, oxidized, mutated) and ubiquitin, into the extracellular space. This led to the speculation that Lewy body contents might contribute to the glial response and the progressive nature of PD. Investigations into the effects of α-synuclein on glial cells have proven to be a step in the right direction in that it is the microglia that seem to be the prime movers in the progressive nature of PD and the demise of the

DA neurons. A number of studies have shown that α-synuclein and its mutants can initiate microglial activation and the ensuing DA neuron loss (Kim and Joh, 2006; Austin et al, 2006; Zhang et al, 2007; Su et al, 2008). The mechanisms involved in the activation of microglia by α-synuclein is the subject of much debate and many experiments. Our cell culture experiments, using BV2 microglial cells, make it clear that α-synuclein as well as modified synucleins can increase microglial cell-to-surface adhesion to both collagen- and laminin- surfaces. We also show that this adhesion is calcium or magnesium-dependant as EDTA significantly blocks adhesion here. Since cell surface receptors mediate microglial adhesion, the fact that we can block the adhesive response with EDTA indicates that integrins may be in play in the microglial adhesive response as these proteins require divalent cations for their action.

Integrins are actually transmembrane molecules that are involved in protein-protein interactions (Shattil et al, 2010). They have an amino acid tail at the outside of a membrane and a single transmembrane domain that connects to a carboxy-terminal cytoplasmic tail. They exist as alpha and beta subunits (Shattil et al, 2010) that communicate with each other. The transmembrane domain is essential for the transmission of information across a membrane. Integrins are thought to be involved in cell-to-cell communication related to the clustering of microglia during adhesion. Our studies on the migration of BV2 microglia show that only LTB4, a leukotriene, and CX3CL1, a chemokine, enticed the microglia to migrate. We need to confirm these results, but we also need to use our cell culture system to elucidate the cascade of events between integrin activation and the production of chemokines and leukotriennes necessary to get to the inflammatory response seen in PD.

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